DEVELOPMENT OF A STRAND-SPECIFIC RT-PCR ASSAY FOR ISAV

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by

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ABSTRACT

Ritchie, R.J. and Gagné, N., 2006. Development of a strand-specific RT-PCR assay for ISAV. Can. Tech. Rep. Fish Aquat. Sci.: iv+27p.

Three diagnostic tools have been developed to test for the presence of the causative agent of Infectious Salmon Anaemia (ISAV): RT-PCR, IFAT, and cell culture. These diagnostic methods are generally in good agreement, but occasionally, a sample will be negative by cell culture or IFAT but positive by RT-PCR. Often these RT-PCR positives are not associated with clinical signs of ISAV. This confounds management of ISAV as it is not clear whether the ISAV identified only by RT-PCR is replicating or if degraded viral RNA was detected, which does not conceivably pose a threat to its host or other fish. To address this question, an assay was designed which can identify by RT-PCR intact, replicating viral RNA. This report details work toward development of a strand-specific RT-PCR assay for ISAV which can be used to detect replicating ISAV.

RÉSUMÉ

Divers outils diagnostiques sont utilisés à l'heure actuelle pour le diagnostique de l'anémie infectieuse du saumon (ISA), soit principalement le RT-PCR, le test d'IFAT, et la culture cellulaire. Ces méthodes produisent des résultats semi-quantitatifs, mais occasionnellement discordants, avec par exemple un résultat positif par RT-PCR mais négatif par culture cellulaire ou IFAT. Souvent, les cas positifs par RT-PCR seulement ne sont pas associés avec les symptômes cliniques de l'ISA. Ceci complique la gestion de la ressource piscicole où l'on cherche à empêcher la propagation du virus. Il est donc important de pouvoir distinguer les poissons porteurs du virus et capables potentiellement de transmettre l'infection de ceux qui sont positifs parce que des débris de virus y sont présents. Ce rapport présente le travail de développment d'un test permettant d'identifier par RT-PCR les brins intacts et réplicants d'ARN viral.

1.0 INTRODUCTION

Since the identification of Infectious Salmon Anemia Virus (ISAV) in North America (Lovely et al., 1999; Bouchard et al., 1999), regulatory agencies, academic institutions, research and diagnostic laboratories alike have been working to characterize the virus and control the disease. Despite the development of three distinct diagnostic techniques to detect ISAV protein (immunofluorescent antibody assays (IFAT), Reverse transcription-polymerase chain reaction (RT-PCR) or infectivity (cell culture)) (Falk and Dannevig, 1995; Mjaaland et al., 1997; Lovely et al., 1999), ISAV has not been eradicated and remains a management concern for regulators.

Discrepancy between results from the different diagnostic tests was an early indication of the complexity of viral etiology, perhaps an early indication of the discrepancy of the techniques, and certainly a complicating factor in disease management. RT-PCR, generally considered the most sensitive of the three assays, often detected the virus in samples which tested negative by IFAT and cell culture. Conversely, on occasion ISAV was not detected using some primer pairs for RT-PCR while other primer pairs gave a positive RT-PCR result (Cunningham & Snow, 2000; Ritchie et al., 2000). The identification of two major ISAV clades (North American and European) went part way to accounting for the absence of RT-PCR positives (false negatives) in the presence of positive test results from the other assays. However, it was not always possible to determine whether the RT-PCR positives were false positives or true positives. The sensitivity of the RT-PCR assay meant that small degraded ISAV RNA, which was unlikely to pose a threat to other fish, and low levels of intact virus which were likely to pose a threat to other fish, could be amplified equally well—if weakly. Indeed, weak RT-PCR positives were posing the biggest problem for disease management as they suggested the presence of low levels of ISAV and were only occasionally supported by positive test results from the two other diagnostic tests (cell culture and IFAT). Further, weak positives often failed to re-amplify possibly due to RNA degradation in storage or due to tissue heterogeneity, hindering confirmation of the initial test result.

With an increasing number of fish testing positive for ISAV by RT-PCR but not confirmed by other diagnostic test, it became necessary to develop a more informative

diagnostic ISAV test. In order to address this issue, investigation into development of a strand specific RT-PCR assay to detect replicating (or living) virus was begun. Cell culture is currently the gold standard for identification of viable ISAV as it assays for ISAV replication and infectivity, however it is not as sensitive as RT-PCR. The new assay was intended to enhance the existing RT-PCR assay—by detecting replicating virus, and to empower regulators responsible for ISAV management.

A strand specific assay has been developed for Hepatitus C Virus (HCV), a single stranded positive RNA virus belonging to the Flaviridae family (Craggs et al. 2001). In this and other positive strand RNA flaviviruses, replication is via the production of a negative stranded replicative intermediate (Westaway, 1987) and detection of negative strand RNA is indicative of viral replication. It was hoped that the converse assay (detection of the positive strand RNA, replicative intermediate) could be developed for the negative stranded ISAV.

It was thought that a strand specific RT-PCR assay for ISAV would allow identification of viable virus by identifying either the replicating strand, via a replicative intermediate, or messenger RNA (mRNA) which are both positive RNA strands and indicative of viable virus (Lamb and Krug, 1996) (Figure 1). The ability to identify replicating virus is particularly important for weakly positive samples and those which are positive by RT-PCR but not cell culture. If the RT-PCR product from these samples arose from degraded ISAV only, they would not possess a replicative intermediate or mRNA and would not be identified as containing positive stranded RNA and would be considered non-viable by this test. Identification of a positive strand RNA (replicative intermediate/mRNA) would thus allow detection of samples containing live virus. These samples and the associated fish could be targeted for management.

The primary objective of this study was to develop a strand specific RT-PCR assay for the detection of replicating ISAV. The secondary objectives were to: i) provide increased information when detecting ISAV at low viral titres and to ii) empower regulators when faced with diagnostic test results. Development of a strand-specific RT-PCR assay involves rigorous testing and optimization of the assay conditions on *in vitro* transcribed RNA followed by testing on field samples. This report details development of the strand specific assay for ISAV.

2.0 MATERIALS AND METHODS

2.1 CONSTRUCTION OF SYNTHETIC ISAV RNA TEMPLATES

ISAV RT-PCR fragments were produced with primers NBFA3 (5'-gag gaa tca gga tgc cag gac g-3') and RA3 (5'-aag tcg atg aac tgc agc ga-3') from segment 8 (modified from Devold et al., 2000) using an ISAV positive RNA clinical NB isolate as template. RT-PCR was performed using Ready-to-go™ RT-PCR beads (Amersham) according to the manufacturer's instructions. The random hexanucleotide-primed reverse transcriptase reaction was amplified with 1.5mM $MgCl_2$ and 0.2uM of each primer for 35 cycles of (94°C-15s/60°C-20s/72°C-1min). The amplicon was cloned into Invitrogen's pCR2.1 according to the manufacturer's instructions. pCR2.1 contains T7 and SP6 promoter binding sites which allow transcription of the insert in both orientations (Figure 2). Colonies were screened by PCR and sequencing to identify constructs containing the amplicons in each orientation.

2.2 PRODUCTION OF SYNTHETIC ISAV RNA

Approximately 1ug of DNA from each plasmid was digested with 5units SpeI in 100ul of 1xSpeI buffer for 3 hours at 37°C. Complete digestion was confirmed by electrophoresis on a 0.8% 1xTBE agarose gel (data not shown). Restriction digest reactions were cleaned by precipitation with 1ul glycogen, 10ul 5M NaOAc (pH5.5) and 260ul EtOH for 2 hours at -20°C. Precipitate was collected by centrifugation for 15 minutes at 14,000 rpm, washed with 70% ethanol and resuspended in 20 ul of DEPC-treated dH_20. DNA concentration was determined spectrophotometrically. RNA was transcribed from the T7 promotor using Ambion's MegaScript™ kit and instructions therein (Figure 2).

2.3 REMOVAL OF PLASMID DNA FROM SYNTHETIC RNA

Plasmid DNA was removed from the transcribed RNA by DNAaseI digestion. The reaction was diluted to a volume of 100ul in 1xDNAseI buffer including 2 units of

DNAseI, and left at 37°C for 1 hour. An aliquot of the reaction was electrophoresed on a 1% 1xTBE gel to check for presence of plasmid DNA. On occasion, two DNAseI digestions were performed (described above) prior to electrophoresis and heat inactivation. The DNAseI digestions were halted by heating at 65°C and the proteins were removed by precipitation with 2 volumes of lithium chloride. Following precipitation at room temperature for 1 hour, RNA was collected by centrifugation at 15,000 rpm at 4°C and washed with 70% Ethanol. Transcribed, 'DNA-free' synthetic RNA was resuspended in 20ul of DEPC-treated water and quantified by spectrophotometry.

Alternatively, or in addition to DNaseI treatment, plasmid DNA was removed or 'degraded' by restriction digest. The sample was digested with 2units of AluI in a volume of 100ul at 37°C for 1 hour. Like the DNAseI treatments, two or more serial restriction digests were performed. RNA was precipitated as above or using one tenth volume 5M NaOAc (pH 5.5) and 2.5 volumes EtOH at -20°C for 1 hour or more. RNA was collected by centrifugation at 15,000 rpm at 4°C and washed with 70% ethanol. Transcribed, 'DNA-free' synthetic RNA was resuspended in 20ul of DEPC-treated water and quantified by spectrophotometry.

The DNA-free nature of the RNA was checked by gel electrophoresis and PCR. Twelve serial ten-fold dilutions of RNA (500ng) were amplified in a 25ul reaction containing 0.25U AmpliTaq Gold®, 1.5mM $MgCl_2$, 0.2uM each primer (NBFA3 and RA3), 0.1mM dNTP, 1 x buffer for 94°C for 10 minutes and 35 cycles of (94°C-15s/60°C-20s/72°C-1min).

2.4 STRAND SPECIFIC cDNA SYNTHESIS / REVERSE TRANSCRIPTION

cDNAs complementary to portions of the positive and negative strand ISAV RNA were synthesized with the tagged primers RA3-tag and NBFA3-tag respectively. The tagged primers contain a 16 nucleotide 'tag' sequence (5'-tca tgg tgg cga ata a-3') unrelated to ISAV at the 5' end whilst the remainder of the primer is ISAV specific. Tagged primers were used on the recommendation of Craggs et al. (2001) in order to reduce the likelihood of mis-priming (Figure 3).

Craggs et al. (2001) showed that high temperature reverse transcriptase increased the strand specificity of the HCV assay, so two different high temperature reverse transcriptases were tested here. For the Tth polymerase (Promega) RNA was incubated with 15pmol specific primer, 200uM dNTP, 1mM $MnCl_2$ and 5 units Tth in 1xTth buffer at 70°C for 2 minutes followed by reduction to 65°C for 30 minutes. The reverse transcriptase activity of the polymerase was inactivated by chelating the Mn^{2+} by addition of 1/10th volume of 10 x preheated chelating buffer (Promega) followed by heating at 98°C for 30 minutes. For the ThermoScript™ Reverse transcriptase (Invitrogen), RNA samples were heated for 5 minutes at 65°C with 15pmol of specific primer and 1mM dNTPs. Samples were placed on ice and the contents were collected by centrifugation. DTT (5mM), 1xcDNA synthesis buffer, 15units ThermoScript RT and 40units of RNAaseOUT™ were added to the tube and the tube was incubated at 60°C for 1 hour. The reaction was terminated by heating to 85°C for 5 minutes.

2.5 FIRST STRAND AMPLIFICATION AND NESTED PCR

Reverse transcription reactions were amplified by PCR or nested PCR. Both reactions consisted of 35 cycles of PCR using a reaction mix containing 0.2uM each primer, 1x PCR buffer (AmpliTaq Gold®), 0.1mM each dNTP, 2.5mM $MgCl_2$ and 1unit AmpliTaq Gold®. The cycle conditions were 8 minutes at 94°C followed by 35 cycles of 94°C for 15 seconds, 60°C for 20 seconds, 72°C for 45 seconds, which was followed by 5 minutes at 72°C. For PCR, one microliter of serial 2 to 10-fold dilutions (depending on starting substrate) of cDNA was added to the first round reaction and amplified with the 'tag' only primer and the gene specific primer opposing the tagged gene specific primer used for the first strand synthesis, i.e. NBFA3-tag primed cDNA was amplified with the tag primer and the RA3 primer and RA3-tag primed cDNA was amplified with the tag primer and the NBFA3 primer (product size 226bp). For nested PCR, one microliter of the PCR reaction was used in a second round of PCR with primers seg8-BF (5'-atg ggc aat ggt gta tgg tat g-3') and seg8-BR (5'-gcg acg atg gcc ttt tct aa-3') (product size 153bp). PCR products were analysed by 11% acrylamide electrophoresis.

2.6 FIELD SAMPLES AND RNA EXTRACTION

RNA samples collected during routine surveillance were used to test the strand specific RT-PCR assay developed. Samples were selected across a range of weak to strong RT-PCR positives, which were or were not associated with cytopathic effects (CPE) in virology, or which had +1 to +3/4 IFAT results. Total RNA had been extracted from these samples previously using Trizol Reagent (Gibco, BRL) as per manufacturer's instructions. The RNA was archived at -20°C prior to use in this study.

3.0 RESULTS

3.1 GENERATION OF CLONED DNA FOR ASSAY OPTMIZATION

Synthetic strand specific ISAV RNA was produced for optimization of the assay by cloning the associated PCR product and transcribing it to the corresponding negative and positive sense RNA strands. The NBFA3/RA3 primers were used to allow incorporation of the strand specific RT-PCR assay developed here into the existing ISAV assay used by diagnostic labs. NBFA3/RA3 primers were used to generate the corresponding amplicon from ISAV infected tissue for cloning into an expression vector. Each amplicon was cloned into the vector in both orientations to allow for RNA transcription using the same enzyme—in this case T7. While it is possible to generate one RNA strand using SP6 and the opposite strand using T7, this entails additional variables between the production of the two synthetic RNAs which was deemed unnecessary and perhaps undesirable. For the segment 8 amplicon, mp13 was designed to produce the negative strand of segment 8 (figure 4a), mp14 the positive strand of segment 8 amplicon (figure 4b). mp 13-transcribed RNA can be primed with NBFA3 only whereas mp14-transcribed RNA (positive strand RNA) can only be reverse transcribed with RA3.

3.2 GENERATION OF SYNTHETIC RNA TEMPLATES

The two vector constructs were used as templates to obtain synthetic RNA corresponding to the negative and positive RNA from the ISAV amplicon. The transcription reaction generated more than ~50ug of RNA (~300nt average size) from 1ug of linearized DNA template. It was estimated that the ratio of single strand RNA of the region of interest to the corresponding region in the plasmid was greater than 1000:1.

Nevertheless, in order to test the strand specific nature of the primers it was necessary to remove the double stranded DNA which contained the target amplicon and could be amplified using the ISAV primers and cause background amplification which would obscure amplification from the strand specific primers. This was done using DNAseI treatment.

Despite multiple attempts to remove the residual plasmid DNA from the synthetic RNA transcripts, DNA was still present following 10^{-2} or 10^{-4} fold dilution of the synthetic RNA following 35 cycles of PCR using primers NBFA3 and RA3 (Figure 5). Following nested PCR, DNA could be detected at 10^{-4} and 10^{-6} dilution of the synthetic RNA (Figure 6). As PCR is known to be a highly sensitive amplification technique the low concentration of DNA, although undesirable was not unexpected. Indeed Craggs et al. (2001) could not achieve complete removal of DNA and had to dilute the RNA over 10,000 times (10^{-4}) to remove all traces of DNA. Like Craggs et al. (2001), multiple attempts were made to remove the residual DNA detectable by PCR. DNAse treatment was doubled (two successive digestions were completed) and DNAse from different manufacturers were tested. In addition, restriction enzymes (AluI) which cut double stranded DNA (but not RNA) were used to render the residual DNA refractory to amplification. Despite the low, persistent level of DNA in the single strand RNA, it was decided to continue with optimization of the strand specific amplification of the reverse transcriptase reaction. Contamination of the synthetic RNA transcripts with plasmid DNA is only an issue in development of the assay and, as no ISAV DNA exists in field samples, the presence of DNA will not be an issue in the final application of the assay.

3.3 TESTING VALIDITY OF THE SINGLE STRAND SPECIFIC REVERSE TRANSCRIPTASE REACTION

Primers were tested using one of two high temperature reverse transcriptases and using RT-PCR and nested PCR. In both cases, NBFA3-tag and RA3-tag were used to transcribe RNA from mp13 (negative strand) and mp14 (positive strand). With both the reverse transcriptases, amplified products were generated from single strand mp13 RNA with NBFA3 *and* RA3-tagged primers, and the same was found for the mp14 RNA. Despite this, and as shown in Figure 7 using Invitrogen's Thermoscript™ reverse

transcriptase, there is a 10^5 to 10^4 difference in specificity between the two primers using the synthetic transcripts—this is comparable to the level of strand specificity seen by Craggs et al. (2001). When nested PCR was used the level of specificity dropped by two orders of magnitude (Figure 8). Thus, for in vivo testing, Thermoscript™ reverse transcriptase was used followed by one round PCR (35 cycles). Tth polymerase gave much lower discrimination levels, in the order of 10^1 and 10^2.

3.4 *IN VIVO* TESTING OF THE STRAND-SPECIFIC REVERSE TRANSCRIPTASE REACTION

In this portion of the study, RNA from a range of strong and weak ISAV field samples were used to study the signal amplified from the positive and negative RNA strands of ISAV segment 8. In each case, two aliquots of 2ug of RNA were amplified from each sample. Each sample was amplified using one of each pair tagged primers using the high temperature reverse transcriptase reaction and PCR. RNA samples classified as weak did not amplify under the conditions used. RNA samples associated with CPE or lack of CPE which previously gave medium to strong RT-PCR amplicons amplified well (Figure 9 & 10). The relative ratios of positive:negative RNA for each sample based on the strength of the amplicon (indicated by the lowest dilution factor at which a product could be seen, e.g. $^{32}/_{16}$ ='2') generated with each of the positive and negative specific primers is shown in Table 1.

4.0 DISCUSSION

A strand specific RT-PCR assay has been developed for ISAV which allows detection of the positive and negative RNA strands of ISAV. The development of the assay described here followed that by Craggs et al. (2001) and benefited from Craggs' research. Taking cues from the previously published work, we used tagged primers and high temperature reverse transcriptase to increase the strand specificity of the reactions. The tagged primers reduced the potential for mis-priming and self priming and the high temperature reverse transcriptase prompted strand specificity. Interestingly, the use of Tth reverse transcriptase lauded by Craggs et al. (2001) gave poor results in this study showing high levels of mis-priming resulting in discrimination levels in the order of 10^1

to 10^2 (data not shown). This is lower than the discrimination levels obtained with Inivitrogen Thermoscript™ and probably due to the temperature at which the RT-PCR was performed. The Invitrogen Thermoscript™ reverse transcriptase was active at 60°C which allowed more efficient transcription using the primers in this study which had annealing temperatures of 60-62°C. The efficiency and specificity of the reaction with Tth could likely be improved by designing primers which anneal at 65°C-70°C, the reaction temperature of Tth polymerase.

Like Craggs et al., *in vitro* testing of the strand specific nature of the primers was dogged by DNA contamination from the *in vitro* transcription of the synthetic RNA templates. The problem of DNA contamination in this type of assay is well documented (Sangar and Carroll, 1998; Craggs et al. 2001; Lerat et al., 1996; Lanford et al., 1994; Lanford and Cavez, 1998). While it is easy to remove the large majority of plasmid contamination from the synthetic RNA as evidenced by gel electrophoresis of the DNaseI treated product, PCR of the RNA templates reveals the presence of DNA, in amounts large enough to hinder easy optimization of the assay. Several attempts were made to reduce the amount of DNA, but as others had done, the strand specificity of the assay had to be determined while working under these conditions. Despite this, extremely convincing levels of strand specificity, similar to those reported by others (10^3 or 10^4 strand specificity, Craggs et al., 2001) were seen.

The strand specific assay was used to amplify RNA from different field samples in the laboratory. All samples had been screened for ISAV and were positive by RT-PCR, IFAT or cell culture. For all samples giving a strong or medium ISAV positive result upon initial screening, the positive RNA strand was amplified at a level higher than the differential (10^4) between each strand specific primer even as determined using synthetic transcripts. This implies that positive strand RNA *is* present in these samples suggesting that they are viable. It was interesting to note that the ratios of positive:negative RNA present in ISAV from CPE and non CPE samples are comparable. This is not what would have been predicted at the start of this study. If lack of CPE is indicative of lack of replication of the virus in the tissue we would have expected to see levels of positive strand RNA below discrimination levels. Instead we see comparable negative and positive levels which are similar to CPE positive samples. This suggests

that the ISAV in the tissue which is associated with both CPE and lack of CPE is replicating and viable. This in turn suggests that lack of growth on cell culture may be due to functional differences between the two viruses. As it is known that different strains with differing abilities to grow on cell culture do exist this result is not surprising, and is certainly worthy of further investigation. The results described here reveal the complex relationship between the RT-PCR and cell culture assays for different samples and suggest that, given the complexity of different ISAV strains, it may be extremely difficult to determine the replicative potential of some strains using conventional methods.

Unfortunately, the samples which were 'weakly' positive on primary screening did not amplify well using this system and these samples, from which information regarding viability is so vitally needed to inform management decisions, did not yield good data. However the reasons for this may be simple and easily corrected in future: in routine RT-PCR up to 40 cycles may be used to amplify the ISAV fragment and samples which amplify 'weakly' in this way may amplify relatively poorly or not at all using the 35 cycle primary PCR described here. Further serial dilution of these samples may not be necessary at all due to their low initial titre. Therefore in future amplification of the 2 strands from weak RT-PCR positives should be assayed using strand specific tagged primers in a 40 cycle primary PCR reaction and the intensity of the two resultant amplicons may be compared by densitometry rather than dilution series.

Because of the sensitivity of the ISAV primers at the core of this assay—it is known for instance that as little as one viral particle can be amplified in 40 cycles—the reasons for the redundancy of the nested PCR are clear. Any benefit the nested PCR may bring in the first few cycles is likely overshadowed by the increased potential for contamination of additional sample manipulation and saturation of the RT-PCR reaction.

As ISAV is a negative RNA virus, the positive strand detected in this assay represents the messenger RNA (mRNA) and also the replicative intermediate (RI). Thus, use of this strand specific assay with ISAV, unlike HCV, will not allow *definitive* detection of the RI as evidence of viral replication. However, identification of relatively labile mRNA or RI (by identification of the positive strand) can be taken as good evidence that mRNA is being produced or that a replicative intermediate exists, which

together suggest that the virus is viable and replicating. The differences between HCV and ISAV genomes and replication strategies underscore the differences in the results of the assay developed for each virus (Craggs et al., 2001). [HCV is a positive strand virus and the positive strand, in addition to being the genomic RNA also serves as a direct template for mRNA. Thus in HCV, the positive strand is the genomic and messenger RNA while the negative strand is the replicative intermediate. The ISAV situation is quite different: here the negative strand is the genomic RNA and the positive strand is the replicative intermediate *and* the messenger RNA].

In the ISAV strand-specific assay, when one assays for the positive strand one is also detecting messenger RNA. Thus, one might expect variation in the ratio of positive to negative RNA following strand specific RT-PCR. In field samples this should not be considered evidence that the level of strand-specificity of the assay developed here is not robust (or specific) enough. Rather it is an indication of the fluctuating levels of positive strand RNA depending on the viral segment being assayed and the virus' infectivity/replicative state. Primers generated from different segments may give different ratios at different stages in the viral life cycle. In a replicating state, one might predict basal expression of a variety of non-structural and capsid genes to generate more viral particles for the new viral RNA to occupy. During infection on the other hand, we might predict high levels of expression of the haemaglutinin gene and genes for other cell surface proteins (Lamb and Krug, 1996). An alternate form of this assay might involve use of T_n primed transcripts which detect only the presence of mRNA, thus indicative of a virus actively expressing proteins for the purpose of replication or infection. However, even if this were to be considered, it would be necessary to have a strand specific assay with which to test the strand specific nature of the Tn primed transcripts to ensure that they contained only positive strand RNA. In either case, it would be useful to set up some phased infections *in vivo* or *in vitro* to study this further and identify patterns which might help application and interpretation of this assay in the diagnostic setting.

The strand specific assay is incredibly important for detection of viable, or replicating, Hepatitis C virus (HCV) as no cell culture system for the propagation of HCV exists. In the case of HCV, the strand-specific assay is a necessity for assaying viral replication (Craggs et al., 2001). By contrast, a well developed cell culture assay

exists for detection of replicating ISAV. One drawback of the ISAV cell culture technique is its failure to detect apparently low viral titres and failure to detect novel strains with functional differences in their surface proteins. Recent ISAV research suggests that there may be a molecular explanation and molecular tests for some of the ISAV strains associated with these phenomena. It is now known that some ISAV variants can not produce CPEs on certain cell lines (Griffiths, et al., 2001, Kibenge et al, 2001; RPC data not shown). There is also evidence that a putative ISAV archetype (possibly benign) strain has been identified which to date does not grow well or not at all in culture (Cunningham et al., 2002; Nylund et al., 2003; data not shown). Thus, while cell culture is still the gold standard for ISAV replication, it can produce false negative results. The strand specific assay developed here can identify replicative or messenger RNA in cell culture negative samples which give a medium to strong band by RT-PCR. The assay is expected to be useful for analysis of the replicative ability in low titre or novel strains using the modified protocol discussed above. However, when assaying for ISAV by any method it is important to be aware of strain differences –many of which can be easily typed by sequencing, PCR-DGGE (Cook et al., 2003) or RFLP—which may affect the assay used results.

The strand-specific RT-PCR assay developed here does have some drawbacks which may affect how and when this assay is used in the diagnostic setting. First the assay is relatively costly to perform. For each sample, two RT-PCR reactions need to be performed (once with each strand specific primer), effectively doubling the cost of the assay, and this cost can be compounded if a dilution series is performed on each sample to study the difference in amplification of the two strand specific reactions. Second, the assay is more time consuming than the standard RT-PCR due to establishment of a dilution series. Third, as the assay is RT-PCR based it is highly sensitive and susceptible to contamination, especially if nested PCR, potentially resulting in false positives. Fourth, the assay is innately difficult to optimize due to DNA contamination prompting questions as to how strand specific the assay really is. In this regard, it is interesting to note that although this method has been published previously (Craggs et al., 2001) for the positive RNA stranded HCV, it has not been used by many others and use of this assay on negative stranded viruses such as ISAV has not been reported. Finally, due to the

huge range in band strength in the ISAV assay, the dilution series must be determined for each samples based on the strength of the RT-PCR assay: a 'strong' RT-PCR positive should be serially diluted 5x or 10x while a 'medium' positive should be serially diluted 2x or 5x and a weak positive should not be diluted at all and should be amplified for 40 cycles. Despite these contraindications, the assay developed here does provide a novel, valuable diagnostic and analysis tool for ISAV. Indeed, recent advances in quantitative RT-PCR (real time or qRT-PCR) may reduce the need to perform a dilution series allowing quantitation in one tube and increasing the feasibility of the assay for routine use. The assay can confirm replication in tissues by detecting the presence of positive strand ISAV RNA in the absence of cell culture (due to strain / culture incompatibility) and will no doubt find a niche in ISAV diagnostics and research.

5.0 RECOMMENDATIONS

The assay developed here should be used to study the replicative status of ISAV in a larger panel of samples. It would be interesting to include in this panel a variety of ISAV strains which are known to grow, or not grow, on cell culture. A modified assay should be considered for weak RT-PCR samples and studied in a panel of weak ISAV positives. In all cases the assay should be studied in conjunction with other diagnostic methods and research methods such as sequencing, and is expected to advance research significantly.

6.0 ACKNOWLEDGMENTS

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7.0 REFERENCES

Bouchard, D., Keleher, W., Opitz, H.M., Blake, S., Edwards, K.C., Nicholson, B.L. (1999) Isolation of infectious salmon anemia virus (ISAV) from Atlantic salmon in New Brunswick, Canada. *Dis. Aquat. Org* 35:131-137.

Cook, M., Vincent, S.L., Ritchie, R.J, Griffiths S.G. (2003) Development of a strain typing assay for ISAV. Bull 1902. Washington, DC: US Department of Agriculture, Animal and Plant Health Inspection Service; US DoI, US Geological Survey; US Dept Commerce, National Marine Fisheries Service.

Craggs, J.K., Ball, J.K., Thomson, B.J., Irving, W.I., and Grabowska, A.M. (2001). Development of a strand-specific RT-PCR based assay to detect the replicative form of hepatitis C Virus RNA. *J. Vir. Meth.* 94:111-120.

Cunningham, C.O. and Snow, M. 2000. Genetic analysis of infectious salmon anemia virus (ISAV) from Scotland. *Dis. Aquat. Org.* 41:1-8.

Cunningham, C.O., Gregory, A., Black, J., Simpson, I., and Raynard, R.S. (2002) A novel variant of the infectious salmon anaemia virus (ISAV) haemagglutinin gene suggests mechanism for virus diversity. *Bull. Eur. Ass. Fish Pathol.* 22(6):366-374.

Devold, M., Krossoy, B., Aspehaug, V., Nylund, A. (2000) Use of RT-PCR for diagnosis of infectious salmon anaemia virus (ISAV) in carrier sea trout Salmo trutta after experimental infection. *Dis. Aquat. Org.* Feb 24;40(1):9-18.

Falk, K. and Dannevig, B.H. (1995) Demonstration of infectious salmon anaemia (ISA) viral antigens in cell cultures and tissue sections. *Vet Res.* 1995;26(5-6):499-504.

Griffiths,S., Mallory, B., Cook, M., and Ritchie, R. J. (2001) Characterisation of ISAV Proteins from Cell Culture. *Dis. Aquat. Org.* 45(1): 19-24

Kibenge, F.S.B., Kibenge, M.J.T., McKenna, P.K., Stothard, P., Marshall, R., Cusack, R.R., McGeachy, S. (2001) Antigenic variation among isolates of infectious salmon anaemia virus correlates with genetic variation of the viral haemagglutinin gene. *J. Gen. Virol.* 82:2869-2879.

Lamb, R.A. & Krug, R.M. (1996) Orthomyxoviridae: The viruses and the replication, (Chapter 21, p605-647) in Fundamental Virology, Third Edition eds. Fields, Knipe Howley et al., Lippincott-Racven Publishers

Lanford, R.E., Sureau, C., Jacob, J.R., White, R., Fuerst, T.R. (1994) Demonstration of in vitro infection of chimpanzee hepatocytes with HCV using strand specific RT/PCR. *Virology* 202:606-614

Lanford, R.E., Chavez, D. (1998) Strand specific rTth RT/PCR for the analysis of HCV replication. In: Lau JYN (ed). Methods in Molecular Medicine, Hepatitis C. Chapter 44. Totowa, NJ: Humana Press Inc.,

Lerat, H., Berby, F., Trabaud, M.-A., Vidalin, O., Major,M., Trépo,C., and G. Inchauspé (1996) Specific Detection of Hepatitis C Virus Minus Strand RNA in Hematopoietic Cells *J. Clin. Invest.*97:3, February 1996, 845-851

Lovely, J.E., Dannevig, B.H., Falk, K., Hutchin, L., MacKinnon, A.M., Melville, K.J., Rimstad, E., Griffiths, S.G. (1999) First identification of infectious salmon anaemia virus in North America with haemorrhagic kidney syndrome. *Dis. Aquat. Org.* 35:145-148.

Mjaaland, S., Rimstad, E., Falk, K., Dannevig, B.H. (1997) Genomic characterization of the virus causing infectious salmon anemia in Atlantic salmon (Salmo salar L.): an orthomyxo-like virus in a teleost. *J Viro* 71(10): 7681-7686.

Nylund, A., Devold, M., Plarre, H., Isdal, E., Aarseth, M. (2003) Emergence and maintenance of infectious salmon anaemia virus (ISAV) in Europe: a new hypothesis. *Dis. Aquat. Org.* 56:11-24.

Ritchie, R.J., Cook, M., Mellville, K., Simard, N., Cusack R., and Griffiths S. (2000) Identification of Infectious Salmon Anaemia Virus (ISAV) in Atlantic Salmon from

Nova Scotia (Canada): Evidence for functional strain differences. *Dis. Aquat. Org.* 44(3):171-178

Sangar, D.V. and Carroll, A. R. (1998) A Tale of Two Strands: Reverse-Transcriptase Polymerase Chain Reaction Detection of Hepatitis C Virus Replication Hepatology, November, p. 1173-1176, Vol. 28, No. 5

Westaway, E.G. (1987) Flavivirus replication strategy. *Adv Virus Res*;33:45-90

8.0 TABLES

Table 1: Summary of field samples tested

Sample	Tissue	RT-PCR	IFAT	culture	Neg/pos
62-f2	Kidney	Strong	+2	negative	2/1
600-f9	Kidney	Medium	+1	negative	1/4
118-f14	Kidney	Strong	Nd.	Nd.	1/1
600-2	Culture	Nd.	Nd.	CPE	5/1
85-1	Culture	Nd.	Nd.	CPE	4/1
1307	Kidney	Pos	Nd.	CPE	1/5
158-f1	Kidney	negative	Nd.	Nd.	Nd.

Neg/Pos indicate the differential between the level of detection of the negative strand compared to the positive strand. For example, 2/1 indicates that twice as much negative strand was present compared to the positive strand. Nd, not done or data not known.

9.0 FIGURES

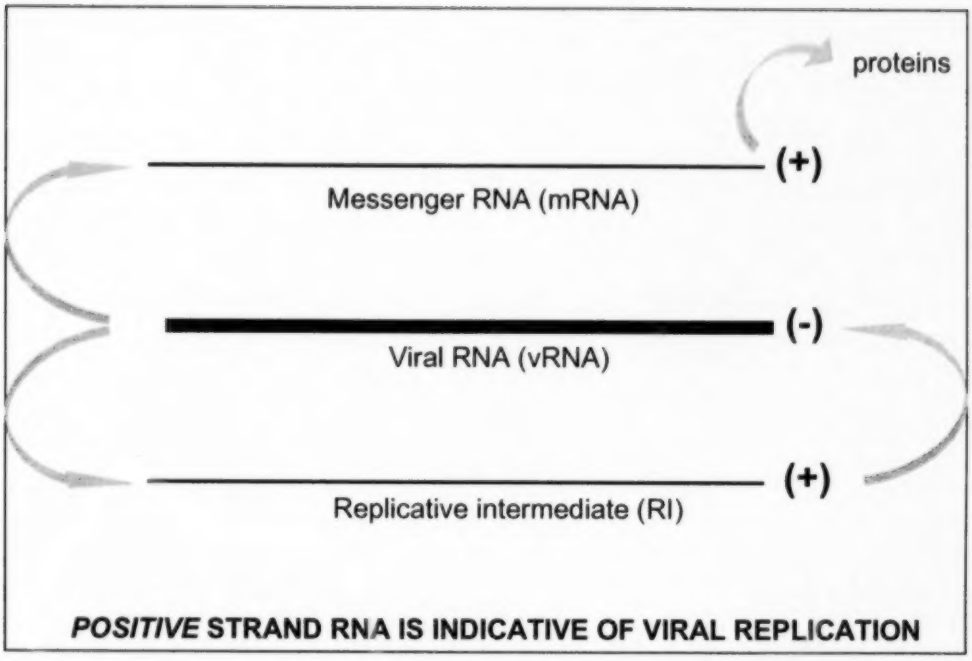


Figure 1. Relationship between the negative and positive RNA strands of ISAV. Schematic representation of the RNA strands associated with the ISA virus. Negative (-) and positive (+) strands are indicated. Curved grey arrows show the relationship between the two strands.

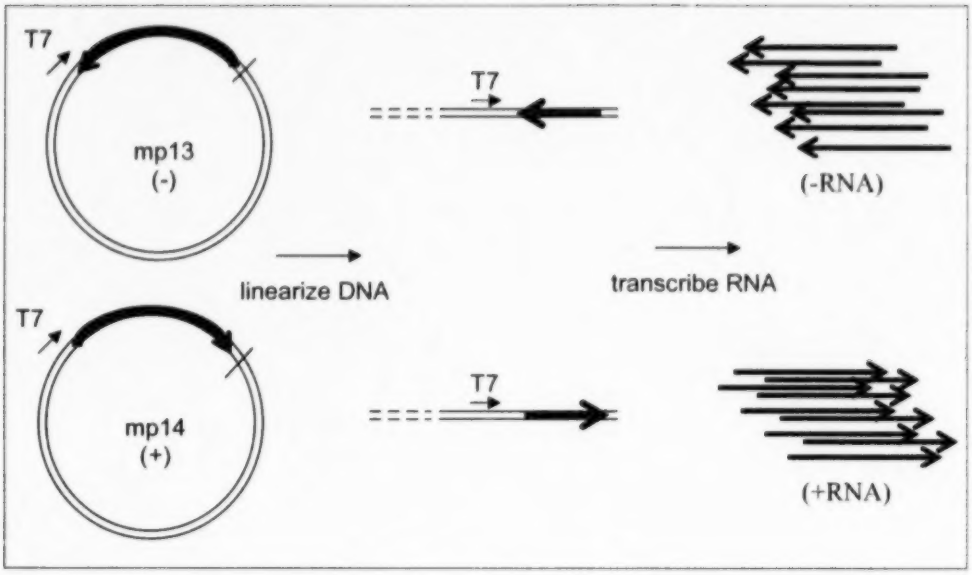


Figure 2. Generation of synthetic ISAV RNA transcripts. Schematic representation of the vectors used to synthesize the synthetic negative (mp13) and positive (mp14) strand ISAV RNA. T7 promoter site is shown in relationship to the ISAV insert (solid box-arrow). The arrow represents the 5'-3' direction. Single line crossing the mp13 and mp14 plasmids represents the *Spe*I site used to linearize the plasmid prior to transcription.

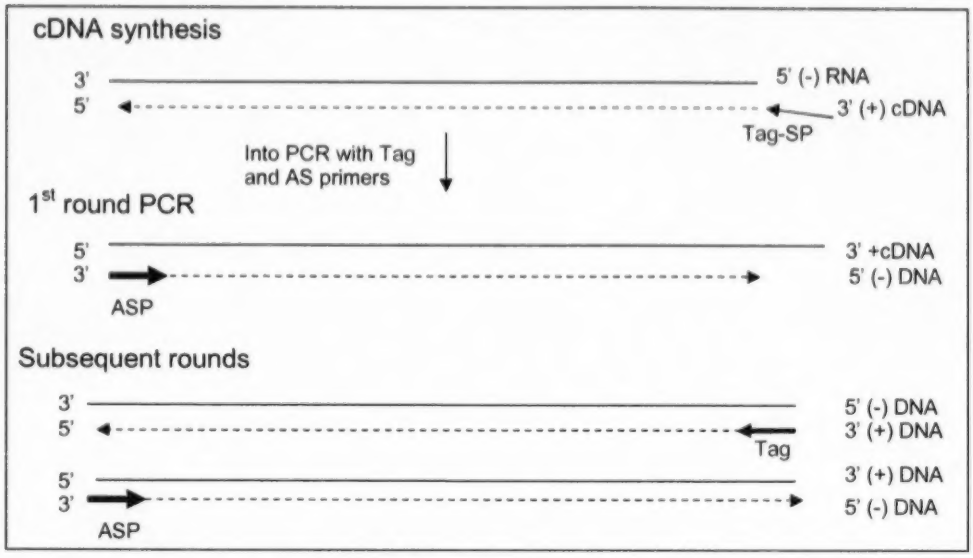


Figure 3. Schematic representation of reverse transcriptase and PCR using tagged primers. ASP-Antisense (AS) primer, SP-Sense primer, Tag-tag primer. Arrow at the end of the dashed line shows direction of elongation/replication. (-) denotes negative or antisense strand, (+) denotes the positive, sense strand.

GACGGCCAGTGAATTG**TAATACGACTCACTATAGGG**CGAATTGAATTTAGC
GGCCGCGAATTCGCCCTTGAAGTCGATGAACTGCAGCGACGATGGCCTTTTC
TAAATTCTCTAGGGCTTTCGAACTTGCACCCAGAGCATACATCTGCATCCTGC
TGTGTAGCATTGTTTTCAGCTCCTTCAGCATCGTCTTCTCCTCTGCCATGTCGG
GTGAAATCATACCATACACCATTGCCCATTTTTCCTCTCCACCATCCG*CGTCCT*
*GGCATCCTGATTCCTC*AAGGGCGAATTCGCGGCCGCTAA

GACGGCCAGTGAATTG**TAATACGACTCACTATAGGG**CGAATTGAATTTAGC
GGCCGCGAATTCGCCCTTG*GAGGAATCAGGATGCCAGGACG*CGGATGGTGGAG
AGGAAAAATGGGCAATGGTGTATGGTATGATTTCACCCGACATGGCAGAGGA
GAAGACGATGCTGAAGGAGCTGAAAACAATGCTACACAGCAGGATGCAGAT
GTATGCTCTGGGTGCAAGTTCGAAAGCCCTAGAGAATTTAGAAAAGGCCATC
GTCGCTGCAGTTCATCGACTTAAGGGCGAATTCGCGGCCGCTAA

Figure 4. Cloning sites and inserts produced for transcription of negative and positive ISAV strands. (Top) Mp13 cloning site and insert for the transcription of negative strand RNA. (Bottom) Mp14 cloning site and insert for the transcription of positive strand RNA. Bold font indicates location of T7 promoter binding site, underline indicates RA3 binding site, italics indicates NBFA3 binding site, boxed font indicates EcoRI site

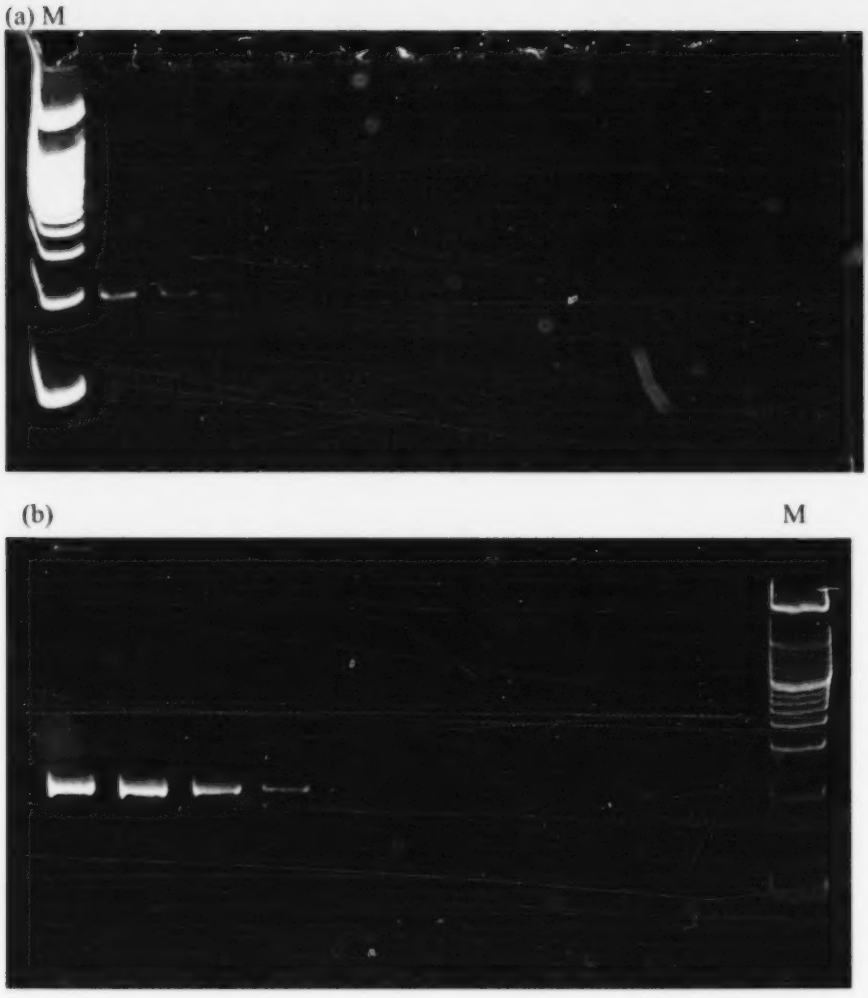


Figure 5: Primary PCR of serially diluted mp13 (a) and mp14 (b) synthetic RNA. Ten times serial dilutions of mp13 (a) and mp14 (b) synthetic RNA amplified in a PCR reaction. M=100bp marker (GibcoBRL). The amplified product runs between the 200p and 300bp size standards. In both gels, the dilution series starts with 10^{-1} on the left hand side of the gel and continues to the right hand side (10^{-12}).

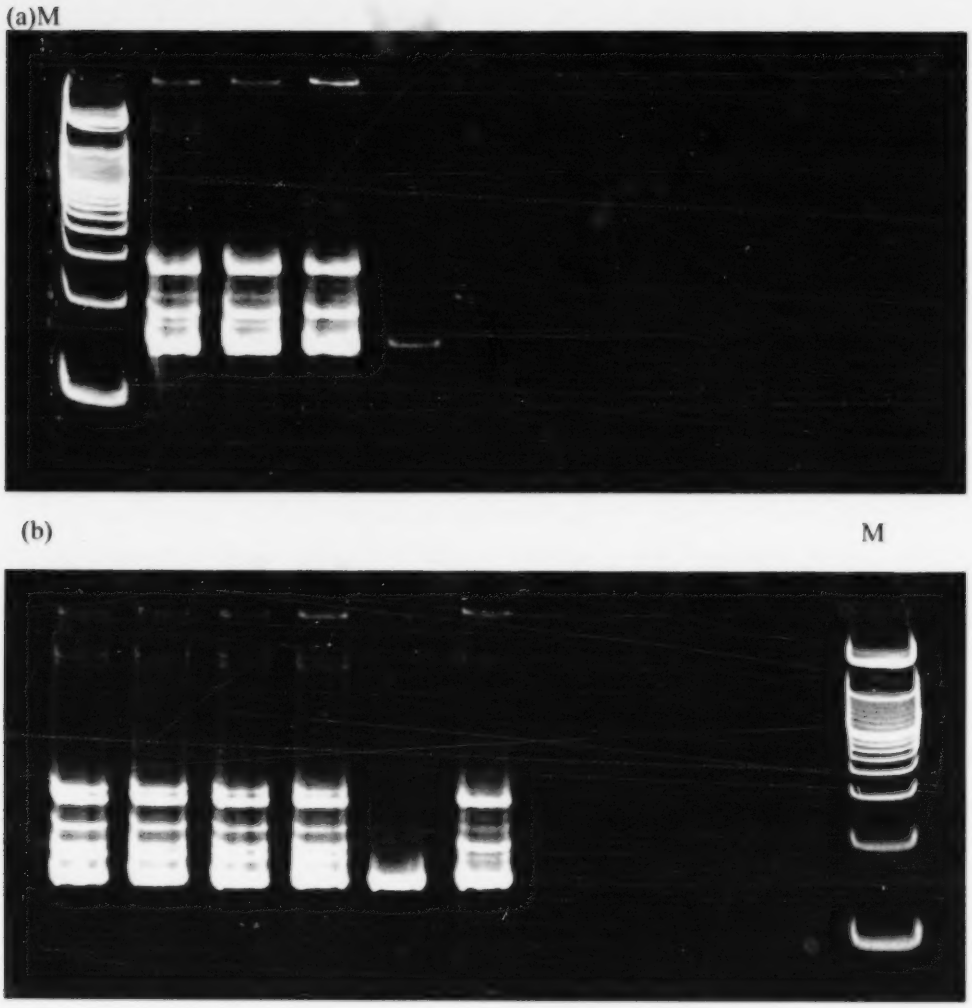


Figure 6: Nested PCR of serially diluted mp13 (a) and mp14 (b) synthetic RNA. Ten times serial dilutions of mp13 (a) and mp14 (b) synthetic RNA amplified in a PCR reaction. M=100bp marker (GibcoBRL). The amplified product runs between the 200p and 300bp size standards. In both gels, the dilution series starts with 10^{-1} on the left hand side of the gel and continues to the right hand side (10^{-12}).

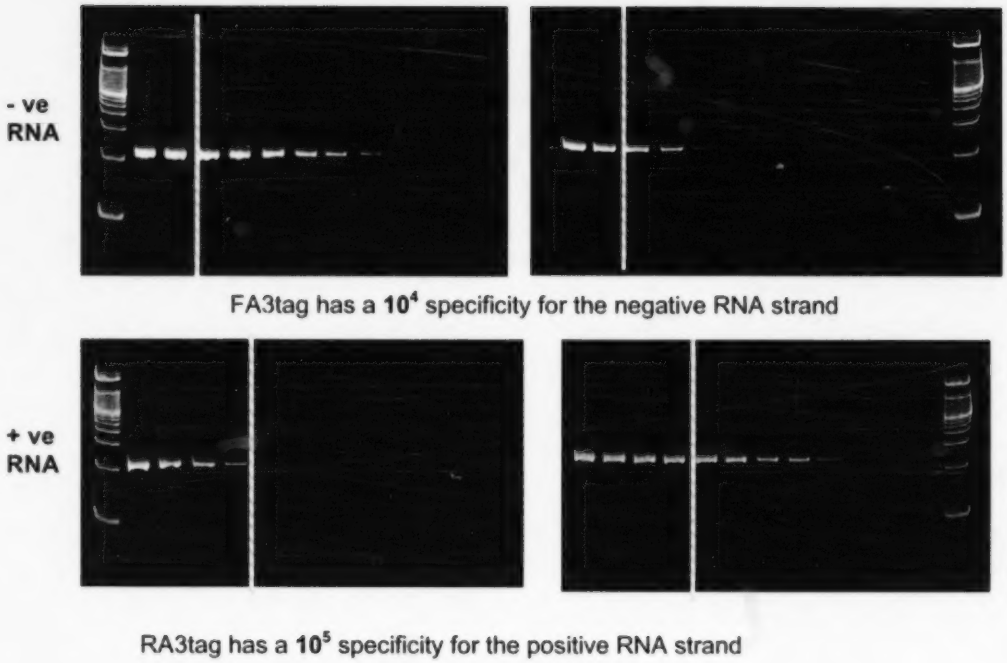

- ve
RNA
FA3tag has a 10^4 specificity for the negative RNA strand
+ ve
RNA
RA3tag has a 10^5 specificity for the positive RNA strand

Figure 7: Primary RT-PCR using strand specific primers on synthetic RNA. Ten times serial dilutions of mp13 negative strand RNA (top two pictures) or mp14 positive strand RNA (bottom two pictures) reverse transcribed with NBFA3-tag primer (left hand side) or RA3-tag (right hand side) and amplified using primary PCR. Dilutions increase from 10^{-1} to 10^{-12} from the left to right of each gel. Light grey, striped vertical line bisecting each gel indicates dilution level at which DNA was detected. M=100bp marker (GibcoBRL).

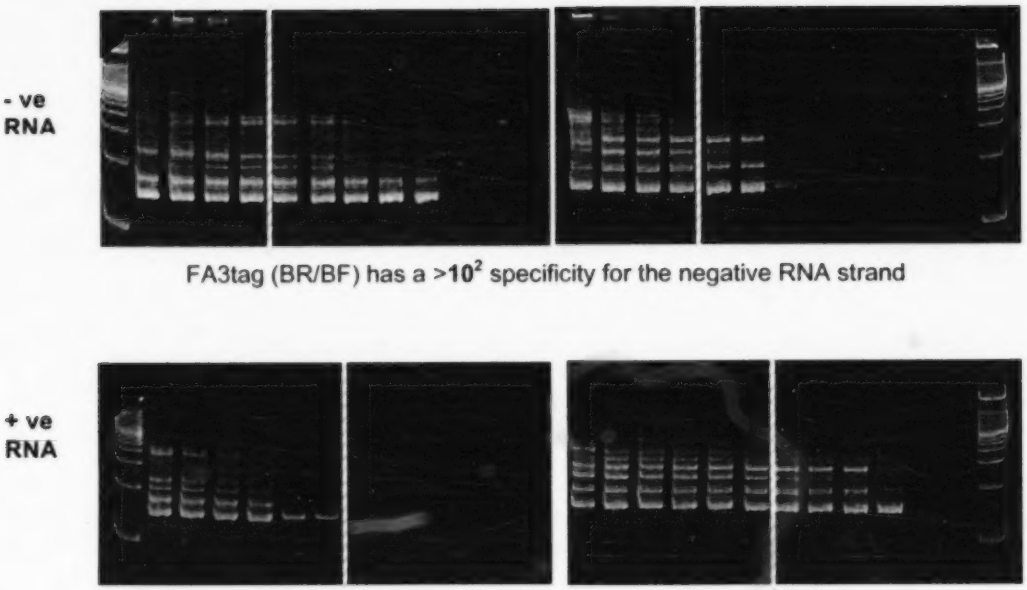

- ve
RNA
FA3tag (BR/BF) has a >10² specificity for the negative RNA strand
+ ve
RNA
RA3tag (BR/BF) has a >10³ specificity for the positive RNA strand

Figure 8: Nested PCR using strand specific primers of synthetic RNA. Ten times serial dilutions of mp13 negative strand RNA (top two pictures) or mp14 positive strand RNA (bottom two pictures) reverse transcribed with NBFA3-tag primer (left hand side) or RA3-tag (right hand side) amplified using nested PCR. Dilutions increase from 10^{-1} to 10^{-12} from the left to right of each gel. Vertical line indicates dilution level at which DNA was detected. Light grey, striped vertical line bisecting each gel indicates dilution level at which DNA was detected. M=100bp marker (GibcoBRL).

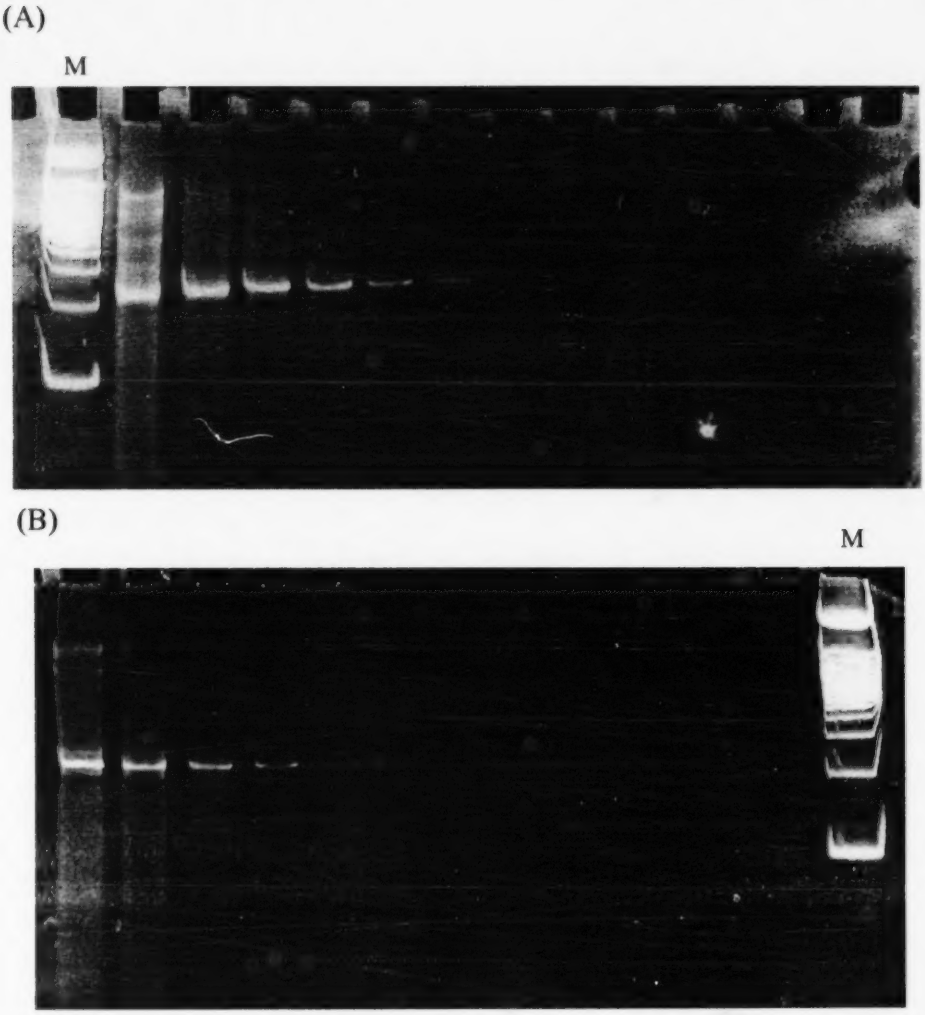
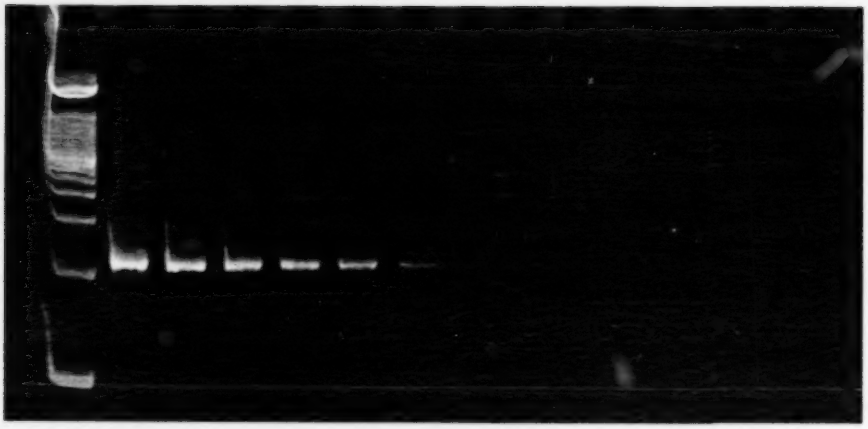


Figure 9: Culture negative/RT-PCR positive field sample. Two fold serial dilutions of NBFA3-tag primed (A) or RA3-tag primed (B) cDNA amplified using primary PCR. The sample (62F2) had previously tested negative for ISAV by culture and positive by RT-PCR. Dilutions increase from 1/2 to 1/4096 from the left to right of each gel. M=100bp marker (GibcoBRL).

(A) M



(B) M

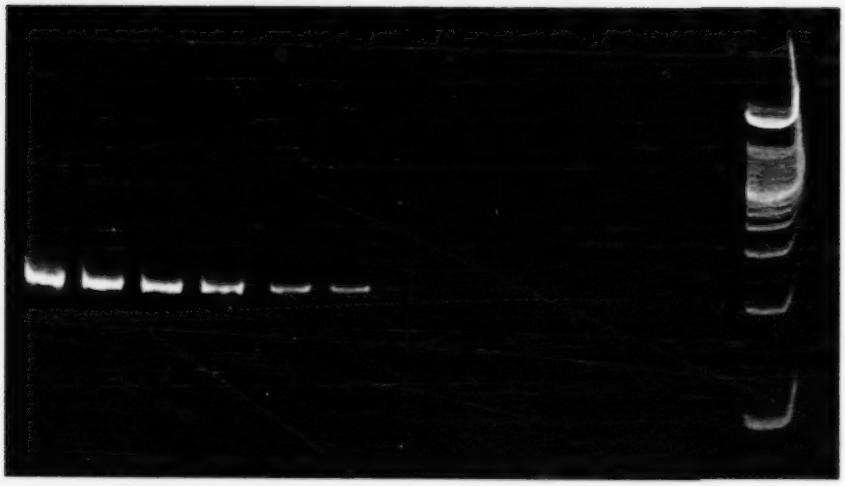


Figure 10: Culture positive/RT-PCR positive field sample. Five fold serial dilutions of NBFA3-tag primed (A) or RA3-tag primed (B) cDNA amplified using primary PCR. The sample (600-2) had previously tested positive for ISAV by culture and negative by RT-PCR. Dilutions increase from 1/5 to 1/244,140,625 from the left to right of each gel. M=100bp marker (GibcoBRL).